Construction of a *Thermococcus kodakarensis* Deletion-Strain Library

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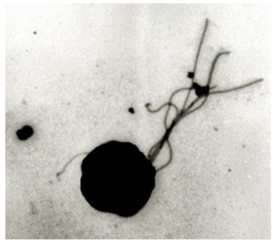
Background knowledge

Thermococcus kodakarensis

- Irregular cocci
- Obligate anaerobe
- Hyperthermophile (opt. growth at 85°C)
- Short (< 1hr) generation time
- ~2x 10^6 base pair genome
- ~2,300 protein genes
- Fermentative heterotroph
- Sulfur as electron acceptor
- Naturally competent for DNA transformation



Thermococcus kodakarensis

Introduction

Deletion-strain libraries have been constructed for Eukaryotes and Bacteria, but not for any member of the third Domain of life, the Archaea. The goal of this project is to construct the first archaeal deletion-strain library, specifically for *Thermococcus kodakarensis*, a hyperthermophilic Archaeon, that is naturally competent for DNA uptake and genetic recombination. The *T. kodakarensis* genome has 2,306 protein-encoding genes [open reading frames; (ORFs)], and completion of this project will result in a library of strains, identical except for the individual deletion of each non-essential ORF. The first step is to construct a library of A-plasmids in Escherichia coli, each one of the 2,306 ORFs cloned individually into plasmid pTS700. Each A-plasmid is then purified from *E. coli* cells and used as the template DNA in a polymerase chain reaction (PCR) that generates a B-plasmid that has the target gene deleted. In the next step, a preparation of each B-plasmid DNA will be used to transform *T. kodakarensis* to generate a *T. kodakarensis* chromosomal deletion strain.

Overall procedure

T. kodakarensis TS559, an agmatine auxotroph (ΔTK0149) that is also resistant to 6-methylpurine ($6MP^R$; ΔTK0664), was constructed as the parental strain for this project. Transformation with plasmid pTS700, and pTS700 derivatives, results in transformants that grow in the absence of agmatine (TK0149 expression) and that are 6MP-sensitive (killed by exposure to 6MP) due to expression of TK0664. Every ORF must first be cloned, with ~500 bp of DNA from the flanking regions into pTS700, generating an A-plasmid from which a B-plasmid is generated with the target gene deleted but with the flanking regions remaining in the plasmid. We now have almost all of the A- plasmids constructed and so are currently focusing on constructing the B-plasmids. Each B-plasmid will be used to transform *T. kodakarensis* TS559 to introduce the deletion into the *T. kodakarensis* genome by homologous recombination (**Figure 1**).

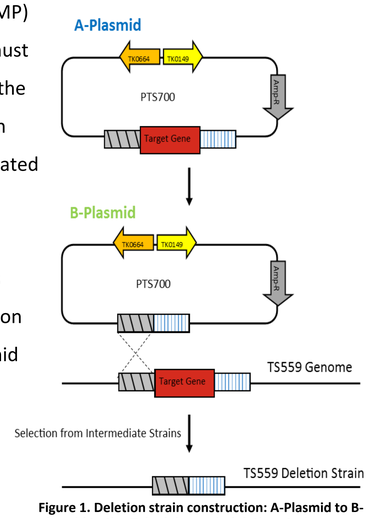


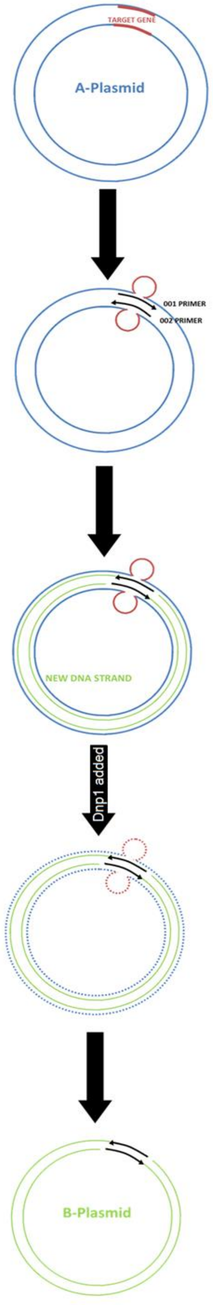
Figure 1. Deletion strain construction: A-Plasmid to B-plasmid to the *T. kodakarensis* deletion strain

Method

B-Plasmid construction

The target gene is deleted from the A-plasmid by an inverse PCR using a QuikChange© site-directed mutagenesis kit (**Figure 2**). The resulting DNA is transformed into *E. coli*, and repaired *in vivo* by *E. coli* recombination enzymes generating a B-plasmid.

Figure 2. Quikchange© PCR procedure



Confirmation of B-plasmid construction

To confirm that the target gene has been deleted, preparations of the A-plasmid and potential B-plasmids are generated from *E. coli* by colony PCR, and their structures compared by agarose gel electrophoresis. A B-plasmid with a deletion migrates faster through the gel than the parental A-plasmid (**Figure 3**).

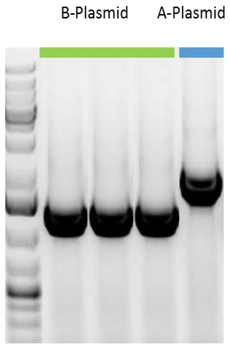


Figure 3. Gel electrophoretic comparison of B- and A-plasmids.

Once identified as a likely B-plasmid, a culture of the *E. coli* strain carrying that plasmid is grown overnight in LB medium, and a preparation of the plasmid then isolated from the resulting cells using a ZR Plasmid Prep Kit©.

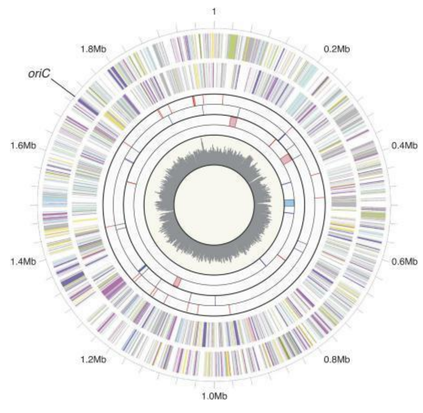
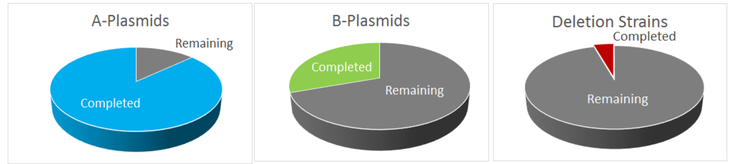


Figure 4. Full *Thermococcus kodakaraensis* Genome.

The precise deletion of the target gene is then confirmed by DNA sequencing. This process is completed for every part of the 2Mbp genome **(Figure 4)**.

Current Project Status

	Constructed & confirmed	Project Completion
A-Plasmids	**2,000**	**87%**
B-Plasmids	**700**	**30%**
Deletion Strains	**100**	**4%**



Conclusion and Future Steps

The importance of this project:

1. This project will establish the first archaeal deletion-strain library
2. The project will identify all essential and nonessential ORFs in *T. kodakarensis*
3. The library will facilitate detailed investigation of all aspects of *T. kodakarensis*
4. The library will be a community resource for all investigations of the common features of Archaea and Eukaryotes.

The deletion-strain library will facilitate research on the unique properties of Archaea and the molecular biology features evolutionarily conserved in both Archaea and Eukaryotes. The deletion-strain library of *T. kodakarensis* will be a foundation and important long-term asset to research and understanding of this Domain.

Acknowledgments and References

Acknowledgments

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References

Hileman TH and Santangelo TJ (2012). Genetic techniques for Thermococcus kodakarensis. Front. Microbiol. 3:195. doi: 10.3389/fmicb.2012.00195

Santangelo, T.J., Cubonova, L., and Reeve, J.N. 2010. *Thermococcus kodakarensis* genetics: TK1827-encoded beta-glycosidase, new positive selection protocol, and targeted and repetitive deletion technology. Appl. Environ. Microbiol. 74:1044-1052.

Fukui T., Atomi H., Kanai T., Matsumi R., Fujiwara S., Imanaka T. Complete genome sequence of the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 and comparison with Pyrococcus genomes. Genome Research. doi: 10.1101/gr.3003105.